



## STAPHYLOKINASE: A BOON IN MEDICAL SCIENCES – REVIEW

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### ABSTRACT

Every year most of the deaths were led by cardio and cerebrovascular disorders around the globe. The use of first or second generation thrombolytics may rise several side effects like bleeding in biological system at the time of treatment. New generation thrombolytics plays an important role in the treatment of thromboembolic disorders which leads to the death. An ideal fibrin specific plasminogen activator, staphylokinase converts a precursor, plasminogen to active enzyme, plasmin to dissolve the blood clot during thrombolytic therapy and it also capable to destroy the normal components of haemostatic system which leads to life threatening consequence and also death. Out of four different thrombolytics which are available in market like alteplase, reteplase, streptokinase and tenecteplase, streptokinase was widely used drug because of its low cost. But, to overcome the hurdles like bleeding and reocclusion efforts have been made to construct the more potent staphylokinase devoid of risk. In this review, progressive development on staphylokinase has been discussed.

**Key words:** Cerebrovascular disorders, thrombolytics, fibrin specific, streptokinase, staphylokinase, reocclusion.

### INTRODUCTION

In Europe and U.S, most of the deaths and disabilities were led by thrombotic diseases, where a thrombus develops in circulatory system can cause vascular blockage [1, 2]. A healthy homeostatic system extensively shows its action at the time of vascular injury to prevent blood loss [3]. Thrombotic disorders like, myocardial infarction, cerebrovascular thrombosis, pulmonary embolism and deep venous thrombolism are life-threatening to humans. Failure of human homeostatic system requires the administration of artificially prepared thrombolytic agents. An attempt with thrombolytic therapy for ischemic stroke was established with plasmin [4] and streptokinase [5].

Plasminogen activators (PA) are characterized into two types: fibrin specific and non fibrin specific. Fibrin specific PA such as tissue-type PA (t-PA) or single-chain urokinase PA (scuPA) and non fibrin specific PA such Streptokinase (SK), two-chain urokinase-type PA (tcu-PA, urokinase) are in clinical use because of its low cost [6]. All these thrombolytics act on thrombus and results the degradation of fibrin.

The use of staphylokinase in thrombotic therapy had a great scope to treatment vascular complications, but it was not reached at the stage of medical practice. From last two decades a progressive research was going on to hunt the ideal thrombolytic drug with minimal risk. So, numerous trails were conducted with the various thrombolytic agents but not reached up to the mark because of cost and specificity. In this review, different properties of staphylokinase, that would appear to have potent thrombolytic agent for treatment of cardio and cerebrovascular disorder.

### STROKE

Every year, approximately 12 million people die of a heart attack or a stroke. It is not limited to the poor as well as rich. Heart attacks and strokes are devoid of sexes. Comparatively women have more risk. Heart gets oxygenated blood through vessels called coronary arteries. When the blood flow to the heart was stopped, it leads to the decreased supply of oxygen results the damage of its function. If the blockage is sudden and onset, it is called "heart attack", if the blockage is fractional and flow of blood to heart was decreased, it can leads to chest pain not permanent damage to heart muscles, but it is a threatening sign to the person in future he/she could alleged from heart attack.

### REASONS BEHIND HEART ATTACK AND STROKES

Heart attacks and strokes are mostly triggered by obstruction of blood flow to heart or brain because of fatty depositions (atherosclerosis) or by the accumulation of blood components on

the inner walls of the blood vessels (blood clots). This makes the flow of blood in vessels was slow and less flexible. So, the blood vessels are unable to supply the blood to heart and brain. Atherosclerosis mainly caused by unhealthy diet, smoking, drinking and chewing of tobacco related compounds.

Strokes can cause in two ways. (i) Blood vessel in the brain can rupture and leads to damaging the brain called intracerebral haemorrhage. (ii) Person has a feeble or uneven heartbeat, clot formation occur in the heart and pass through the blood vessels to the brain. These clots trapped in a narrow brain artery and blood flow was blocked to the brain.

### IDEAL FEATURES OF THROMBOLYTIC DRUGS

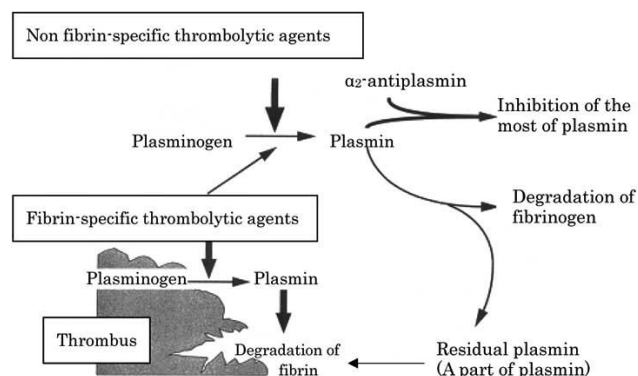
- Fibrin specificity
- Stability
- Good patency.
- No reocclusion rate
- Resistant to plasminogen activator inhibitor-1(PAI-1)
- Non antigenic
- Cost effective
- Easy to Administer
- Low frequency of systemic bleeding and intracranial hemorrhage

### THROMBOLYTIC AGENTS

Thrombolytics were used to treat different types of venous and arterial thromboembolic disorders [7]. These are the plasminogen activators (PA's) that make use of the vascular system's own intrinsic thrombo resistance defense mechanism by accelerating and amplifying the conversion of an inactive plasminogen to the active plasmin, a natural fibrinolytic agent, which in turn hydrolyzes several key bonds in the fibrin (clot) matrix causing dissolution [8]. The detailed mechanism of fibrin specific and non fibrin specific thrombolytics were shown in Fig – 1.

Thrombolytic drugs would have preferably rapid reperfusion can establish TIMI grade 3 flow in patients alleged by thrombosis [9, 10]. These agents would also have extended half-life that permits single bolus dosing, facilitate more timely and problem-free administration. Thrombolytic agents can be categorized on the source of the agent, the tendency for enhanced enzymatic activity on a fibrin or cell surface or based on the mechanism of action or different generations wise. Each of these methods of classification is useful in helping to characterize the diverse nature of plasminogen activators, but regardless of how one defines these agents, they all serve one primary purpose i.e the conversion of plasminogen to plasmin. Each of these approved agents for

clinical use has been briefly discussed below with their drawbacks also.



**Fig 1: Working mechanism of the non-fibrin specific thrombolytics and the fibrin-specific thrombolytic agents. The thickness of the arrow indicates the efficacy of the action. The fibrin-specific thrombolytic agents more efficiently dissolve the thrombus than the non fibrin specific thrombolytic agents (Source from Current Pharmaceutical Design, 2006, 12, 849-857).**

### UROKINASE

A 411 amino acid serine protease, Urokinase, the first generation molecule is a non fibrin specific thrombolytic agent and also called urokinase-type Plasminogen Activator (u-PA) was produced from human urine, having the molecular weight of 54 kDa. It consists of three domains (a) Carboxyl terminal serine protease domain (b) Kringle domain (c) Amino terminal, a growth factor domain [11]. Severe side effects were also associated upon the administration of urokinase to the patients alleged from acute myocardial infarction. Due to these reasons FDA detached the Abbokinase® (Brand name of urokinase) from the market because of major deviation from current good manufacturing procedure.

### STREPTOKINASE

Wonder drug, Streptokinase is an plasminogen activator produced by various strains of  $\beta$ -hemolytic Streptococci. In 1933, streptokinase fibrinolytic activity was described [12] and in 1958 it was used in the patients with acute myocardial infarction and these results changed the focus of treatment. It is a non-fibrin specific extracellular enzyme with molecular weight of 47 kDa composed of 414 amino acids, make use of its fibrinolytic action activating the circulatory plasminogen indirectly [13]. Like urokinase, streptokinase has three distinct domains, (a).  $\alpha$  (residues 1–150) (b).  $\beta$  (residues 151–287) (c).  $\gamma$  (residues 288–414) with half life ~ 30 min. It does not exhibit plasmin activity but binds to plasminogen in 1:1 ratio and shows plasmin activity [14]. Being a prokaryotic protein, it is allied with allergic reactions and also antibody-mediated inhibition of plasminogen activation. Because of its non fibrin specific SK was also associated with bleeding complications and rethrombosis.

### RECOMBINANT TISSUE PLASMINOGEN ACTIVATOR

A serine protease (tPA) is a fibrin specific thrombolytic molecule consisting of 527 amino acids with a molecular weight of 70kDa (15). Tissue plasminogen activators available in two forms (a). single chain form (sct-PA) (b). two chain form (tct- PA). It contains four domains (a). A fibronectin type I finger (F) domain with NH<sub>2</sub>-terminal region of 47-residues (residue 4 to 50), (b). An epidermal growth factor domain (residue 50 to 87), (c) Two kringle domains comprising residues from 87 to 176 and from 176 to 262 (K1 and K2) (d). A serine proteinase domain (residues 276 to 527) with active site residues His 322, Asp 371 and Ser 478 [16]. Plasminogen activator inhibitor- 1(PAI-1) inhibits the PA activity of t-PA [17]. The amino acids at 296-299 in t-PA was associated with the inhibition by PAI-1 [18]. In the absence of fibrin, t-PA is a poor

enzyme to activate the plasminogen. But in the presence of fibrin it enhances the plasminogen activation rate.

Alteplase, a tissue plasminogen activator was produced by r-DNA technology. It is synthesized using the cDNA of human melanoma cell line [19]. COOL 1 and COOL 2 clinical trail's demonstrated the efficiency and safety of t-PA in the treatment of central venous access device (CVAD) occlusion [20]. In GUSTO-1, patients treated with alteplase had a 6.3% mortality rate and 0.72% incidence of intracranial haemorrhage [21]. Compared with streptokinase, the second generation thrombolytic molecule, alteplase shows 1% drop in death rate or nonfatal stroke. After the onset of stroke symptoms, alteplase was given as I.V to the patients within 3 hours and most common and severe side effect with alteplase was bleeding.

### APSAC

Anisoylated plasminogen-SK activator complex (APSAC) is the another form of SK. The equimolar acylated complex of human lysplasminogen and SK act on plasminogen upon deacylation spontaneously in plasma. Activation of anistreplase to lys-plasminogen-streptokinase was occurs by hydrolytic process in blood or thrombus. The hydrolytic process results the release of p-anisoyl group by deacylation. After intra venous administration of anistreplase, a non-enzymatic deacylation process was started and it converts plasminogen to which the streptokinase is bound [17]. On single bolus administration, The International Study of Infarct Survival (ISIS)-3 showed a 10.5% mortality and 0.6% intracranial hemorrhage [22]. The brand name of anistreplase was Eminase® and possesses the side effects like streptokinase.

### TENECTEPLASE

Tenecteplase is a t-PA mutant of alteplase and was bioengineered to have extended half-life. Asn<sup>117</sup> was substituted by Gln results the deletion of glycosylation site in kringle 1 domain. Substitution of Thr 103 with Asn reintroduces the new glycosylation site. Replacement of the amino acids Lys<sup>296</sup> - His<sup>297</sup> - Arg<sup>298</sup> - Arg<sup>299</sup> with Ala increases the resistance to PAI-1. The deletion of carbohydrate chain at Asn<sup>117</sup> prolongs its half-life 17-20 min. Like native t-PA, tenecteplase also binds to fibrin [23, 24]. The ASSENT-1 (assessment of safety and efficacy of a new thrombolytic agent) trial showed the safety and efficacy of tenecteplase [25] and ASSENT-2 trails in patients with acute myocardial infarction shows bleeding and allergic reactions associated by using this drug [26].

### RETEPLASE

Reteplase (recombinant plasminogen activator, r-PA) is a derivative of t-PA, consists of 355 amino acids with a molecular weight of 39 kDa. It is a unglycosylated protein with single chain deletion variant of alteplase expressed by *Escherichia coli*. Like alteplase, in the absence of fibrin it has plasminogen activator activity, but the binding affinity to fibrin is significantly (5 fold) lower than alteplase [27]. The competence of reteplase in patients with acute myocardial infarction has been evaluated by RAPID I (recombinant plasminogen activator angiographic phase II international dose finding study) trial, where it was administrated as a double bolus against to the standard dose of alteplase, the patency of reteplase was reached earlier and more frequently than with alteplase. Like alteplase, in the GUSTO (global use of strategies to open occluded coronary arteries) III trial the mortality rate after 30 days was not significantly different with reteplase as a double bolus [28]. Like other drugs, bleeding and allergic reactions are also associated by using the reteplase.

All the above mentioned thrombolytics posses some side effects mainly bleeding complications and reocclusion. By keeping these problems, now – a – days a lot of research is focused on third generation molecule staphylokinase.

### STAPHYLOKINASE

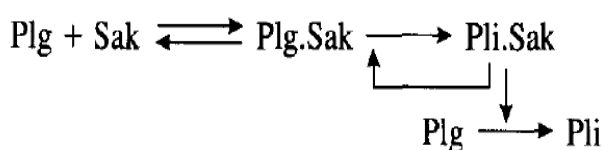
Staphylokinase is a bacterial protein found in the culture medium of many strains of *Staphylococcus aureus* that converts inactivate plasminogen into active proteolytic enzyme plasmin [29, 30] shown to have profibrinolytic properties more than five decades

ago [31]. Davidson [32] and Glanville [33] precipitated staphylokinase from culture supernatants by adjusting the pH to 3.3 with 10M - HCl. Glanville precipitated the staphylokinase with 75 % (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and it was purified by chromatography on CM-cellulose columns or by affinity chromatography on plasmin – Sepharose [34] or on plasminogen – Sepharose [35]. The *in vitro* fibrinolytic properties of staphylokinase was evaluated [36, 37] and *in vivo* thrombolytic properties were studied in dogs [38, 39]. Staphylokinase – neutralizing activity was detected by using a clot lysis assay [40].

Staphylokinase gene encodes a total of 163 amino acids, where the first 27 amino acids are coding for the signal peptide and from amino acid 28 corresponding to the full-length mature staphylokinase (mSAK) [41, 42, 43, 44, 45] as a single polypeptide with molecular weight of approximately 15.5 kDa [46] and shows no significant homology with streptokinase [47].

SAK does not have any enzymatic activity and activation of plasminogen was performed by two step mechanism [48]. Initially formation of complex between SAK and plasmin(ogen). Later the SAK – plasmin(ogen) complex active site is exposed with the conversion of plasminogen to plasmin, thus SAK acting as a potent plasminogen activator. In the initial lag phase, plasminogen in SAK-plasminogen complex is activated to plasmin with the trace amounts of plasmin, which later contaminates in plasminogen preparation [49]. The complex between streptokinase and plasminogen exposes the active site of plasminogen devoid of any proteolytic cleavage [50]. After generation of some plasmin, SAK binds to plasmin rather than plasminogen and then SAK-plasmin complex directly activates plasminogen to plasmin significantly (Figure – 2). PA activity of SAK-plasmin (ogen) complex was inhibited by α2-AP in the absence of fibrin [51, 52]. The inhibition by α2-AP was suppressed by EACA, lysine binding site (LBS) of plasmin in this complex [53] and inhibitory effect was reduced in the presence of fibrin or FCB-2 by competing for interaction with the LBS [54]. The initial rate of plasminogen activation by SAK was accelerated two to three folds by the addition of fibrin [55]. SAK does not bind to fibrin and the enzymatic efficiency of the plasminogen activation by SAK was improved 30-fold, 38-fold and 8.5- fold by the (DD)E complex [56]. So, SAK-plasmin(ogen) complexes express fibrin-specific plasminogen activation on the surface of fibrin and degrades fibrin (Fig – 2).

The following model was proposed for the activation of plasminogen (Plg) by staphylokinase (Sak).



**Fig 2: Activation of plasminogen (Plg) by staphylokinase (Sak) (Source from Blood, 1994, 84: 680-686)**

SAK was highly fibrin specific but having lesser fibrinolytic properties when compared to streptokinase. Systemic fibrinogen degradation, α2-antiplasmin consumption, and plasminogen activation were not found with the use of staphylokinase. The plasma half life of staphylokinase was 6.3 min in phase I trials. Double-bolus administration was safe when compared to single bolus administration [57]. In the CAPTORS (Collaborative Angiographic Patency Trial of Recombinant Staphylokinase) I and II trials the therapeutic potential of SAK and its derivatives has been studied and support its therapeutic potential [58, 59].

Clinical results with staphylokinase was encouraging, but heterologous protein staphylokinase is immunogenic in humans and induces antibody formation, not possible to repeated administration and its potency is limited [60]. 38% of treated patients was alleged by rethrombosis [61]. To reduce the immunogenicity and prolonged half-life of SAK, site directed

mutagenesis had been done without the considerable loss of their fibrinolytic and fibrin specificity. In order to reduce the immunogenicity and prolonged half-life of wild type SAK, polyethylene glycol-derivatized cystein substitution variants of recombinant SAK (Code SY161-P5) was generated by substitution of 12 amino acids in SAK molecule (K35A, E65Q, K74R, E80A, D82A, T90A, E99D, T101S, E108A, K109A, K130T, K135R) reduced antigenicity.

#### GENE STRUCTURE OF STAPHYLOKINASE

A 489 bp sequences of full length staphylokinase encodes signal peptide followed by 163 amino acid mature staphylokinase. The coding sequence was preceded upstream by canonical Shine – Dalgarno sequence and by -10 and -35 prokaryotic promoter sequences. This 136 amino acid staphylokinase shows no significant homology with streptokinase. The four nucleotide difference between the coding regions of *sak*ØC, *sak*42D and *sak* – STAR genes because of silent mutations. These affect the codons for amino acids in full length staphylokinase at 38, 61, 63, and 70 (in mature staphylokinase 11, 34, 36, and 43) [43, 62, 63, 64]. The four affected condons (a) Amino acid 38 is Lys in all staphylokinase moieties (b) Amino acid 61 is Ser in Sak STAR but Gly in SakØC and Sak42D (c) Amino acid 63 is Gly in Sak STAR and in *Sak*ØC, but Arg in Sak42D (d) Amino acid 70 is His in SakSTAR and in *Sak*ØC, but Arg in Sak42D. All the reported sequences were markedly in the 3' region of the staphylokinase molecule, approximately 160 nucleotides downstream of the stop codon were suggesting that this region is not important for protein expression.

#### PROTEIN STRUCTURE OF STAPHYLOKINASE

X-ray scattering, dynamic light scattering, ultracentrifugation, and UV circular dichroism spectroscopy revealed the solution structure of staphylokinase. Radius of gyration, stokes radius, maximum dimension and sedimentation coefficient was 2.3 nm, 2.12 nm, 10 nm and 1.71 S respectively indicated the shape of staphylokinase is elongated. Sak contains two folded domains of similar size and the mean distance of the centers of gravity of the domains is 3.7 nm. The positions of the two domains are variable in solution, signifying that the molecule is like a flexible dumbbell [65]. Based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE]) and iso-electric point Several molecular forms of staphylokinase was purified with different molecular weights [66, 67, 68]. Low molecular derivatives of mature staphylokinase were obtained lacking the 6 or the 10 amino terminal amino acids. In buffer milieu, on interaction with plasmin(ogen) mature staphylokinase is converted to Sak-Δ10 and it was shown to have same fibrinolytic activity like SAK. Amino acid at 26 was useful for the activation of plasminogen by staphylokinase, substitution with Arg or Val results the loss of functional activity, but substitution with Leu or Cys has little or no effect on functional activity [69]. In mutant protein these amino acid changes doesn't affect the solution structure. Three-dimensional structure of the molecule will be required to know these observations at molecular level.

#### EXPRESSION OF STAPHYLOKINASE FROM BACTERIA

With the help of recombinant DNA technology and molecular techniques, staphylokinase gene was isolated from lysogenically converted *S. aureus* and expressed in non-pathogenic expression hosts *E.coli*. The *sak* gene is cloned into pET-28a+ and expressed in an IPTG inducible *E.coli* BL21 and salt induced *E. coli* GJ1158. The mature recombinant *sak* protein expressed in *E.coli* is also further extracted and analyzed by different methods to demonstrate thrombolytic activity.

In *Escherichia coli* BL21 the expression of recombinant staphylokinase has led to the formation of inclusion bodies and the final yield of the protein was 20 mg/L [70], 200 mg/L fermentation broth [71], 300 mg/L [72], 70 to 500 mg/L [73], 1 g/L [74] and 2.8 g/L of fermentation broth [75]. Also, the *sak* expression was carried out in different expression host systems like *E.coli* [76, 77, 78], *Bacillus subtilis* [79], *Streptomyces lividans* [80].



## EXPRESSION OF STAPHYLOKINASE FROM YEAST

Methylotrophic yeast *Hansenula polymorpha* provides an alternative expression system and has successfully developed for several commercial production of protein and in vaccine production. The supernatant of fermented rSAK-1 strain was treated with endoglycosidase H led to removal of the smear-like band, while the well-defined band at 16 kDa was non-glycosylated rSAK-1. This provided the evidence of rSAK-1 strain secretes both glycosylated and non-glycosylated rSAK-1, while the rSAK-2 strain secretes only nonglycosylated rSAK-2. Amino acid sequences of wt-SAK and SAK-variants of rSAK-1 (= THR 174) and rSAK-2 were shown below. The difference in the primary structure of the amino acid sequence of three SAK molecules were shown in *italics* and bold letters indicates the potential N-linked glycosylation-site motif. Thr-30 was replaced with Ala in rSAK2 strain for nonglycosylated protein [74, 81].

### wt SAK

SSSFDKGGKYYKGGDDASYFEPTGPYLMVNVTVGDSKGNELLSPH  
YVEFPIKPGTTLTKEKIEYYVWALDATAYKEFRVVELDPSAKIE  
VTTYDKNKKKEETKSFPIKTEKGFVWDLSEHIKPNPGLNITKVVIE  
KK

### rSAK1

SSSFDKGGKYYKGGDDASYFEPTGPYLMVNVTVGDSKGNELLSPH  
YVEFPIKPGTTLTKEKIEYYVWALDATAYQEFVVSLSPSAKIE  
VTTYDKNKKKEETKSFPIKTEKGFVWDLSEHIKPNPGLNITVYVIR  
KK

### rSAK-2

SSSFDKGGKYYKGGDDASYFEPTGPYLMVNVAGVDSKGNELLSPH  
YVEFPIKPGTTLTKEKIEYYVWALDATAYQEFVVSLSPSAKIE  
VTTYDKNKKKEETKSFPIKTEKGFVWDLSEHIKPNPGLNITVYVIR  
KK

The recombinant *Pichia* carrying multiple insertions of SAK gene yielded high-level (~1 g/l) of extracellular glycosylated rSAK (~18 kDa) (74). Addition of antibiotic like tunicamycin during the induction phase results the expression of non-glycosylated and highly active rSAK of ~15 kDa. Staphylokinase was also produced as heterologous fusion protein in methanol inducible *Pichia pastoris* GS115 and has the good fibrinolytic activity [82].

## STAPHYLOKINASE WITH THROMBOLYSIS AND ANTIPLATELET ACTIVITY

Based on site-directed mutagenesis, Arg-Gly-Asp (RGD) motif was engineered in the staphylokinase (SAK). This mutant of SAK was designated as RGD-SAK, was expressed, purified and characterized. Biochemical analysis revealed that RGD-SAK has similar structure and fibrinolytic function like SAK. In *in vitro* platelet binding activity RGD-SAK has higher affinity with platelets than SAK and *in vitro* platelet-rich clot lysis assay demonstrated that the engineered mutant outperformed the non-manipulated SAK. Time and the concentration required to obtain 50% platelet rich clot lysis ( $C_{50}$ ) were reduced significantly when using different concentrations of RGD-SAK compared with SAK. RGD-SAK was found to inhibit ADP-induced platelet aggregation where the SAK had negligible effect on platelet aggregation. RGD-SAK had the dual function to target the platelet-rich clots and to block platelets aggregation. It may serve as a more potential thrombolytic agent with platelet-targeted thrombolytic and antiplatelet aggregation activities in compared with SAK. Addition of RGD may decrease the rethrombosis. Table 1: revealed the  $K_m$  and  $K_{cat}$  of both SAK and RGD-SAK.

**Table 1: Kinetics of equimolar mixture of human plasmin with RGD-SAK.**

Thrombolytic agent	$K_m$ ( $\mu M$ )	$K_{cat}$ ( $s^{-1}$ )	$K_{cat}/K_m$ ( $\mu M^{-1} s^{-1}$ )
SAK	0.39 ± 0.03	0.015 ± 0.002	0.033 ± 0.003
RGD-SAK	0.78 ± 0.05	0.031 ± 0.001	0.037 ± 0.005

## SAK VARINATS

Recombinant RGD-hirudin by fusing the tripeptide RGD sequence to the native hirudin (wt-hirudin) and was expressed at high levels in *Pichia pastoris* [83]. The anti-thrombin activity of purified recombinant RGD-hirudin and sak-hirudin was 12,000 ATU/mg, which is equivalent to native – hirudin, but r-RGD-hirudin only inhibit platelet aggregation. The biological effects of r-RGD-hirudin and r-sak-hirudin on Thrombin Time (TT), Prothrombin Time (PT), Activated Partial Thromboplastin Time (APTT), Bleeding Time (BT), maximum platelet aggregation (PAGm) induced by ADP were studied in animal models and compared with that of wt-hirudin. r-sak-hirudin and r-RGD-hirudin was three times more effective in contrast to wt-hirudin in preventing thrombosis. The animals were infused r-RGD-hirudin had prolonged TT, PT, and APTT which were similar to that of wt-hirudin; but only r-RGD-hirudin was capable of inhibiting PAGm.

Different methods of statistical optimization were carried out on different media components concentration for enhanced production of sak variants having maximum fibrinolytic activity [84].

## CHIMERIC MULTIFUNCTIONAL STAPHYLOKINASE

Chimeric staphylokinase was created by cross linkage of staphylokinase with hirudin to antifibrin and antiplatelet antibodies. Now a days, research studies proved that activated platelets play a key role in arterial thrombosis and rethrombosis. After thrombolytic therapy the secondary clots were formed by the action of platelet aggregation [85]. RGD tripeptide can recognize the platelet membrane glycoprotein IIb/IIIa (GPIIb/IIIa) receptor and binding of surface glycoprotein GPIIb/IIIa to fibrinogen mediates platelet aggregation and RGD prevents fibrinogen binding to GPIIb/IIIa on activated platelets, thus inhibiting platelets aggregation activity [86]. It indicates the clot lysis efficacy of SAK can be dramatically enhanced with the ability of direct active platelet binding and rethrombosis complication can be minimized with antiplatelet aggregation.

Several chimeric proteins have been produced based on the attempts to combine the advantages of staphylokinase with thrombin inhibitor and Platelet aggregation inhibitor to prevent reocclusion. Hirudin was selected as antithrombin partner due to its compatibility with the host secretion system. A model fusion protein was constructed by fusing the small functional domain hirudin variant (HV1) in both N- and C- terminals of sak results the HV1-SAK and SAK-HV1. Plasminogen activation of SAK was not altered by the addition of molecules at N- terminal or C- terminal sequences. But at N- terminal cleavage with lysines results the unstable fusion. However, C- terminal fusions represent stable configuration for rational development of improved thrombolytic agents based on SAK [87].

SAK and hirudin were joined together via a pair of engineered coiled sequences that act as the hetero dimerization domain. A lysine rich coiled coil sequence (K-coil) is added to the C-terminal tail of SAK to generate SAK-K coil (SAKK). Glutamic acid rich coiled coil sequence (E-coil) is added to the C-terminal end of hirudin to generate hirudin-E. Coli (HE). These heterodimeric molecule (HE-SAKK) is a superior thrombolytic agent in comparison to staphylokinase as confirmed by *in vitro* fibrin and plasma clot lysis studies [88]. Site directed mutagenesis at K35 was substituted with Arg (R) and results in novel SAK variant RGD-SAK has higher affinity with platelets than SAK and possesses thrombolytic activity and ADP-induced platelet aggregation in a dose dependent manner [89]. PLATSAK (Platelet-Anti-thrombin-Staphylokinase) was designed which include three inhibitory regions RGD sequence, a pair of fibrinopeptide A, an inhibitor of thrombin and the C- terminal of hirudin a direct anti-thrombin [90].

In an another approach of improvement of the thrombolytic potential of SAK, as well as to introduction of antithrombotic and antiplatelet activity, the recombinant SAK-RGD-K2-Hir and RGD-K2-Hir-SAK were constructed which were the more potent and fast acting thrombolytic agent as compared with standard r-SAK [91, 92].

## CONCLUSION

Thrombolytic therapy remarkably reduces the mortality and morbidity caused by acute myocardial infarction and also used in the treatment of various other thromboembolic disorders. Because of the complication while in use i.e bleeding, fibrin specificity and reocclusion, researchers focussed their attention on third generation thrombolytic molecule with minimal side effects. Patients will fail to achieve and complete reperfusion with the current regimens. For improved efficacy and safety profile, genetically engineered 'third generation' thrombolytic agents are improved clinical and also the opportunity to determine the relative importance of fibrin specificity, plasma half-life, and resistance to inhibition by plasma inhibitors in thrombolytic therapy. In addition antibody targeted plasminogen activators and antithrombin agents are in use to construct the thrombolytic variants. However, the optimal thrombolytic strategy has yet to be determined which have the capability of ~100% reperfusion and without complications like bleeding and reocclusion. In depth research studies are going to develop the perfect thrombolytic agents devoid of side effects.

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